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Semiconductor nanocrystals for biological imaging

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Conventional organic fluorophores suffer from poor photo stability, narrow absorption spectra and broad emission spectra. Semiconductor nanocrystals, however, are highly photo-stable with broad absorption spectra and narrow size-tunable emission spectra. Recent advances in the synthesis of these materials have resulted in the generation of bright, sensitive, extremely photo-stable and biocompatible semiconductor fluorophores. Commercial availability facilitates their application in a variety of unprecedented biological experiments, including multiplexed cellular imaging, long-term *in vitro* and *in vivo* labeling, deep tissue structure mapping and single particle investigation of dynamic cellular processes. Semiconductor nanocrystals are one of the first examples of nanotechnology enabling a new class of biomedical applications.

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Introduction

Semiconductor nanocrystals (see glossary), also called quantum dots (QDs), are a new class of fluorescent biological labels. Originating from quantum confinement (see glossary) of electrons and holes within the nanocrystal core material, the fluorescence from QDs is unique compared with that from traditional organic fluorophores. For example, QDs exhibit high photostability, broad absorption, narrow and symmetric emission spectra (see glossary), slow excited state decay rates and large absorption cross sections (see glossary) [1]. Their emission color can be continuously tuned from ultraviolet to visible and infrared wavelengths by changing the size and chemical composition of the semiconductor core nanocrystal. Growing a semiconductor shell with a larger band gap (see glossary) improves the quantum confinement, resulting in very bright and highly stable, chemically as well as optically, semiconductor fluorophores [2,3]. QDs offer an exciting potential to overcome many of the limitations encountered by traditional organic dyes and genetically engineered fluorescent proteins. Since their introduction into biological imaging in 1998 [4,5], an enormous body of research has emerged focusing on the synthesis, photophysical property characterization and bioconjugation [6–9] of QDs. Advanced molecular and cellular imaging with QDs has also been realized [10,11•].

Biocompatible QDs find utility not only as a basic biolabeling tool but also as a key building block for complex multi-functional bio-probes. Their large surface area can be tailored to bind both target selective molecules and therapeutic molecules, enabling efficient delivery of treatments via a probe to the diseased area. Complex nanostructures formed by linking QDs and gold nanoparticles through DNA hybridization or streptavidin–biotin interaction have also been realized [12–14] and applied in sensing bio-molecular concentrations [14]. Although QDs have been utilized in a broad range of imaging applications to date, their versatility for advanced biomedical applications remains to be fully explored. In this article, we focus on the applications of semiconductor nanocrystals in biological imaging, with a brief introduction into their unique optical properties, followed by a discussion of recent progress in both *in vitro* and *in vivo* imaging and their applications in neurobiology. Toxicity issues are also addressed.

Optical properties

The size-dependent optical properties of QDs are the result of their quantum-confined electronic states [15]. Similar to the 'particle in a box' model, excitons (see glossary) in smaller nanocrystals experience stronger quantum confinement, resulting in larger photoluminescence energy. Figure 1 shows the typical excitation and emission spectra (see glossary) of water-soluble QDs. Their emission wavelength can be continuously tuned from 400 nm to 2000 nm by changing both the nanocrystal size and their composition [16].

In contrast to conventional fluorophores, QDs have broad absorption, narrow and symmetric emission spectra. These features enable concurrent imaging of multiple entities in a single biological experiment, this is a difficult task with standard fluorophores because their relatively narrow excitation and broad emission spectra often result

Glossary

Absorption spectrum: The spectrum depicting the wavelengths of electromagnetic radiation absorbed by a sample. The absorption pattern is unique and can be used to identify the sample.

Absorption cross section: A measurement of the ability of an atom or molecule to absorb light at a specified wavelength.

Acinus (plural form: Acini): In this review, acinus refers to the smallest division of breast tissue, which is the sac-like part of the milk producing glands.

Autofluorescence: Also known as natural fluorescence or self-induced fluorescence, which arises from substances such as favins, porphyrins and chlorophyll in biological systems.

Band gap: The energy difference between the top of 'valence band' and the bottom of 'conduction band' in a semiconductor material.

Defocused microscopy: A technique to study defocused images of samples with a light microscope.

Emission spectrum: A type of fluorescence spectrum that illustrates fluorescence evoked over a range of wavelengths when the incidence light wavelength is constant. (From Wikipedia encyclopedia, http://en.wikipedia.org/).

Excitation spectrum: A type of fluorescence spectrum that illustrates fluorescence produced over a range of wavelengths of the incidence light. (From Wikipedia encyclopedia, http://en.wikipedia.org/).

Exciton: An exciton is a bound state of an electron and a hole in a semiconductor, or in other words, a Coulomb correlated electron–hole pair. (From Wikipedia encyclopedia, http://en.wikipedia.org/).

Hela cell: One of the cells grown from the cervical cancer of a young African-American woman, Henrietta Lacks. Hela cells were the first immortalized human cell line.

Neurotransporter: High-affinity transport proteins that are located in the plasma membranes of both presynaptic nerve and presynaptic glial cells. Neurotransporters mediate the removal of neurotransmitters from the synaptic cleft and function as intracellular transport systems that concentrate neurotransmitters in synaptic vesicles.

Particle in a box: The particle in a box is a simple, idealized system that can be completely solved within quantum mechanics. It is the situation of a particle confined within a finite region of space (the box) by an infinite potential that exists at the walls of the box. The particle experiences no forces while inside the box, but is constrained by the walls to remain in the box. (from Wikipedia encyclopedia, http://en.wikipedia.org/).

Photostability: Refers to the rate at which molecules degrade to both UV and visible light.

Quantum confinement: Light emission from bulk (macroscopic) semiconductors, such as LEDs, results from exciting the semiconductor either electrically or by shining light on it, creating electron-hole pairs which, when they recombine, emit light. The energy, and therefore the wavelength, of the emitted light is governed by the composition of the semiconductor material. Because the physical size of the semiconductor nanocrystals is considerably reduced to be much smaller than the natural radius of the electron–hole pair, additional energy is required to 'confine' this excitation within the nanoscopic structure, leading to a shift in the emission to shorter wavelengths. (from Glossary, http://www.qdots.com).

Quantum yield: The ratio of the amount of light emitted from a sample to the amount of light absorbed by the sample.

Red tail: Refers to the long tail on the red (long wavelength) end in an asymmetric fluorescence emission spectrum.

Semiconductor nanocrystals: Also called quantum dots, or artificial atoms. They are semiconductor crystals with diameters of a few nanometers. They are made from a variety of different compounds, and hence are referred to as II-VI, III-V or IV- semiconductor nanocrystals, based on the periodic table groups that the elements are from. For example, cadmium selenide nanocrystals are II-VI semiconductor nanocrystals because they each contain an element cadmium (Cd) from the periodic table group II and an element selenide (Se) from the periodic table group VI. The electronic energy levels in semiconductor nanocrystals are discrete, and the spacing of the electronic energy levels can be precisely tuned through variation of their sizes and components.

Stokes shift: The difference between the peak absorption and the peak emission wavelengths.

Θ and Φ: Angles used in spherical coordinates. In this review, the z-axis corresponds to the optical axis. Θ and Φ are used to determine the orientation of an individual dipole. Θ is the zenith angle from the z-axis with a range from 0 to π, and Φ is the azimuthal angle in the xy-plane from the x-axis ranging from 0 to 2π.

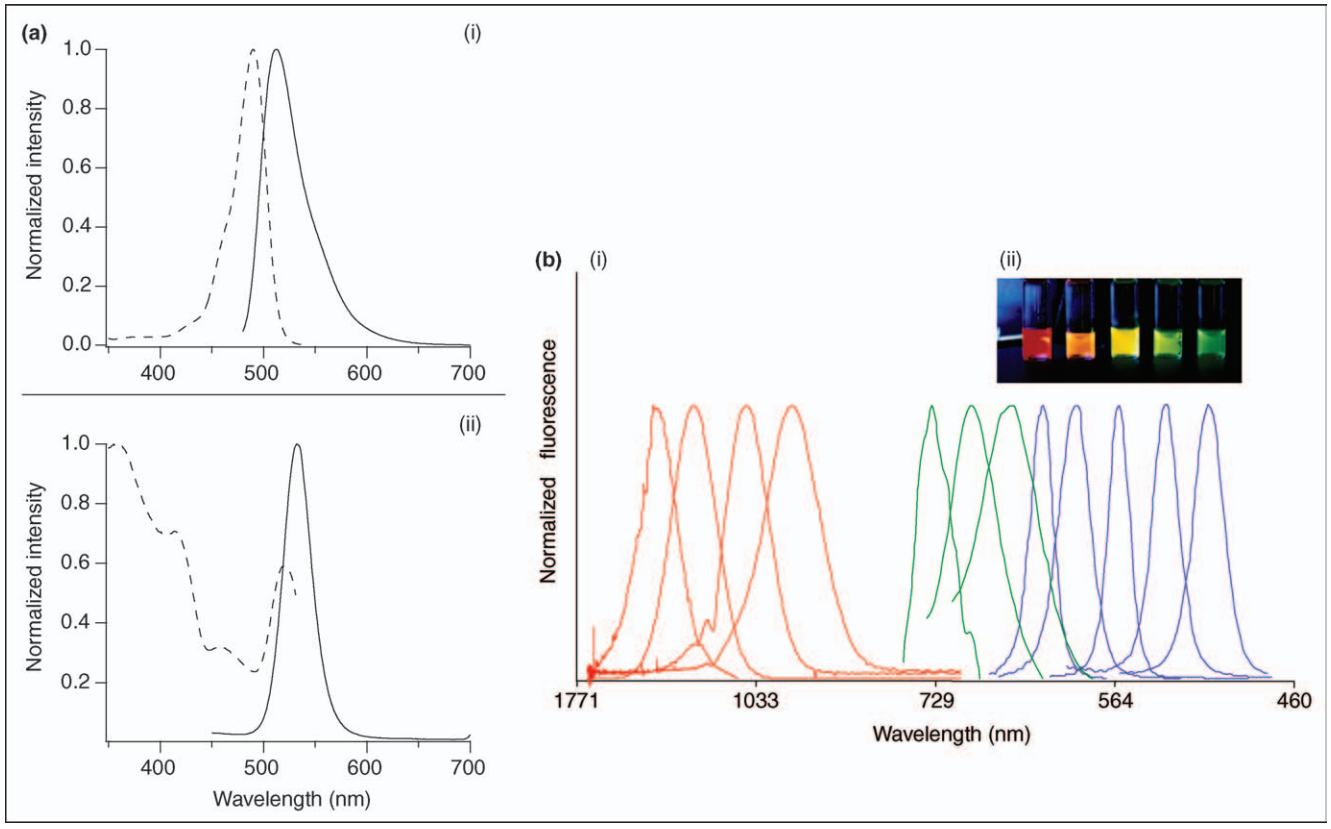
in spectra overlap [2]. Another advantage of QDs is that they are highly resistant to metabolic degradation and are hundreds of times more photo stable than conventional fluorophores. In addition, QDs often have a large Stokes shift (see glossary), that is, a large separation between the excitation wavelength and the emission maxima, this has the effect of reducing autofluorescence (see glossary), resulting in a several-fold increase in sensitivity versus that of organic fluorophores [17].

For QDs, quantum yields (see glossary) can be as high as 89% at room temperature [18]; molar extinction coefficients, about 10^5–$10^6\ M^{-1}\ cm^{-1}$, are 10 to 100 times larger than those for most organic dyes [19]; and they have two-photon absorption cross sections that are several orders of magnitude larger than those of organic dyes [20]. Optical properties of QDs are usually unaffected by conjugation to bio-molecules. Thus, they are both highly stable and bright probes, especially suitable for photon-limited *in vivo* studies and continuous tracking experiments over extended time periods. A more extensive discussion of the photophysical properties of QDs is presented by Grecco *et al.* [21].

In vitro imaging

QDs have been very successful tool for immunofluorescent labeling. With the continuous effort that has gone into developing high quality biocompatible QDs, nanoparticles conjugated to antibodies, peptides and DNA have been prepared and used to target specific tissues and cells, therefore enabling multiplexed labeling and long-term studies that cannot be undertaken with standard dyes [22,23••,24–29]. Although QDs and organic dyes can have comparable quantum yields, the larger absorption cross-section of the nanocrystal results in a much stronger photoluminescence signal. The sustained strong signal from a single nanoparticle has been used to track dynamic cellular processes over time scales that are not viable using organic fluorophores [10]. Recently, Dahan and co-workers [30] developed a method to study single nanocrystal fluorescence patterns using defocused microscopy (see glossary). By relating these patterns to the structures of the nanocrystal emission dipoles, the experimenters were able to determine the three-dimensional orientation of the nanoparticles, and successfully applied this technique to track the orientation of single membrane receptor in live cells (Figure 2). With continuous efforts in

Figure 1

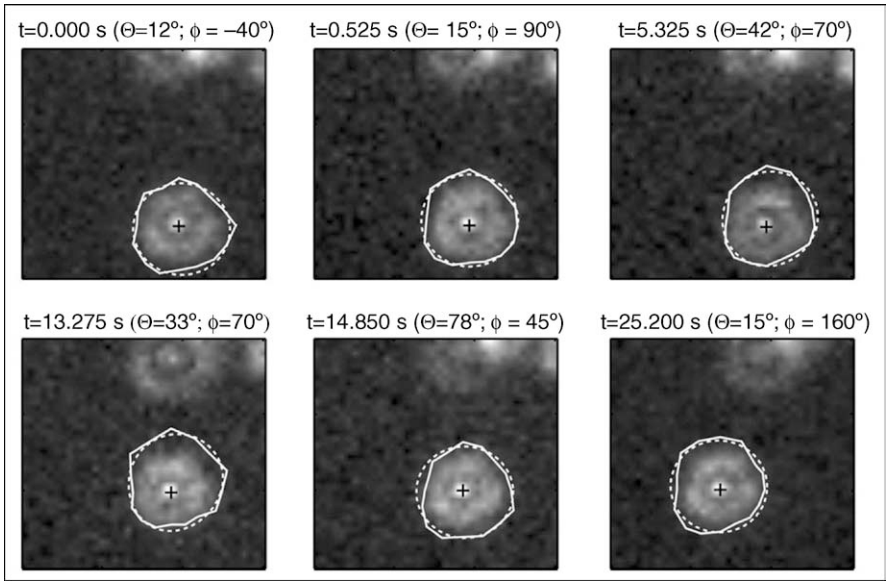


The typical excitation and emission spectra of QDs. **(a)** Excitation (broken) and fluorescence (solid) spectra of **(i)** fluorescein and **(ii)** a typical water-soluble QD sample in phosphate buffered saline (PBS). The nanocrystals have a much narrower emission (32 nm compared with 45 nm at half maximum and 67 nm compared with 100 nm at 10% maximum), no red tail (see glossary) and a broad, continuous excitation spectrum (arbitrary units on y-axes). **(bi)** Size- and material- dependent emission spectra of several surfactant-coated QDs in a variety of sizes. The blue series represents different sizes of CdSe QDs with diameters of 2.1, 2.4, 3.1, 3.6 and 4.6 nm (from right to left). The green series represents InP (indium phosphide) QDs with diameters of 3.0, 3.5, and 4.6 nm. The red series represents InAs (indium arsenite) QDs with diameters of 2.8, 3.6, 4.6, and 6.0 nm (arbitrary units on y-axis). **(bii)** A true-color image of a series of silica-coated core (CdSe)-shell (ZnS or CdS) nanocrystal probes in aqueous buffer all illuminated simultaneously with a handheld ultraviolet lamp. Reproduced with permission from Bruchez *et al*. [4].

elucidating the photophysics of single QDs, there will be increasing interest in their application as fluorescent emitters for studying dynamic biophysical processes. For example, Yildiz and Selvin [31] demonstrated that total internal reflection microscopy used in conjunction with organic fluorophores can produce fluorescence imaging with one nanometer accuracy (FIONA). This technique involves a large number of photons being collected over time from a single dye molecule, enabling researchers to locate the center of the fluorescent pattern with high precision. FIONA has been applied to unravel the walking mechanism of the molecular motors myosin V, myosin VI and kinesin. Although the presented experimental results were from an organic dye, the authors believed that using QDs would provide at least 10-fold improvement in time resolution and are extending the applications of FIONA in motor movements with QDs.

QDs can be taken up by live cells nonspecifically, possibly because of the characteristic size range and good biocompatibility of the QDs. Pellegrino *et al*. [32] studied the phagokinetic tracks left on a homogenous layer of QDs, and demonstrated that QDs can be used as a two-dimensional *in vitro* invasion assay for discriminating between non-invasive and invasive cancer cell lines. This technique provides a new tool for qualifying tumor cell invasiveness. Internalized QDs are also powerful probes for long-term studies of cell–cell interactions. They have been used to examine the interactions of human mammary epithelial tumor cells with normal cells that were growing in a 3-D culture system. The tumor cell behavior around polarized normal cell clusters was clearly demonstrated when tumor cells and normal cells were labeled with nanocrystals of different emission colors. The high photostability (see glossary) of the QDs is crucial in the tracking and imaging of these co-cultures for extended

Figure 2



Defocused microscopy images of QD coupled glycine receptors in the membrane of a Hele cell. The contour intensities (broken lines) can be fitted (solid lines) to determine the orientation (Θ, Φ) of each QD (see glossary). Here, the experimenters show a proof-of-principle experiment that defocused microscopy can be used to probe the rotational dynamics of single biomolecules. The cross is the origin in the spherical coordinates of a defocused pattern, t stands for time. From this series of defocused images of the same cell at different time points with distinct azimuthal and zenith angles, experimenters tracked the orientation of individual QDs coupled to a glycine receptor in the membrane of a Hela cell (see glossary). Reproduced with permission from Brokmann *et al*. [30].

time periods (up to 14 days) and cannot be replaced by organic fluorophores (Figure 3, from RM Boudreau, unpublished).

Using semiconductor nanoparticles for *in vitro* labeling enables fluorescent and electron microscopy imaging of the same probe [10,18,33], so that information on both temporal dynamics and high-resolution cellular localization can be obtained [10]. The fluorescence and electron density properties of QDs were also utilized by Nisman *et al*. [33] to label a nuclear protein on cell sections and to correlate the fluorescence and transmission electron microscopy (TEM) data. They also employed QDs in conjunction with immunogold to co-localize proteins at the ultrastructural level. Moreover, by obtaining cadmium elemental maps of the core shell CdSe–ZnS QDs distributed on a nuclear structure, the authors demonstrated the potential of using quantum dots as tags for electron spectroscopic imaging to co-localize multiple proteins [33].

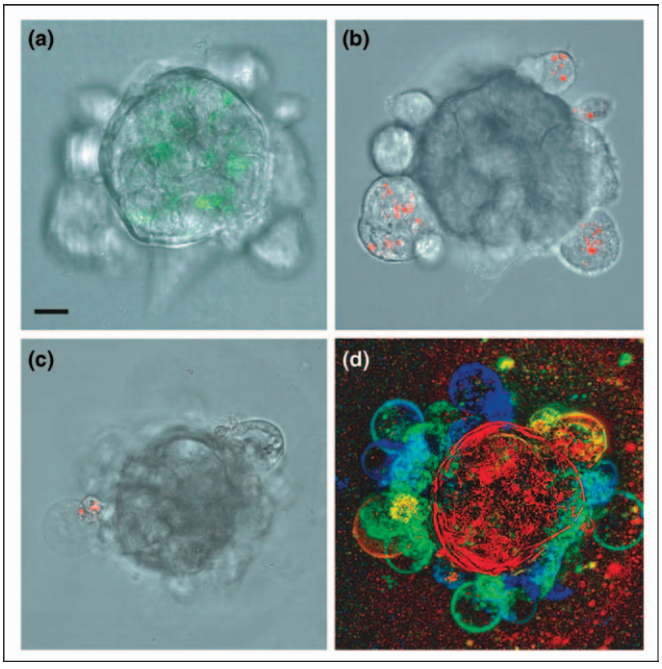
In vivo imaging

The extreme brightness of QDs and their resistance to photobleaching enable continuous exposure under laser illumination for an extended period of time, making them especially useful for *in vivo* imaging. Progress in nanocrystal synthesis, coating and surface modification has significantly enhanced their applications in tracking and imaging. Efforts to optimize the surface coating for *in vivo* imaging have shown that specific polyethylene glycol (PEG) coatings result in longer circulation time [18,34•], enhanced stability [18] and minimal nonspecific deposition [18,35], which are essential elements for *in vivo* imaging.

Although the first *in vivo* targeting experiment imaged the histological sections of mouse organs after intravenous injection of peptide conjugated QDs, recent applications primarily focus on live animal imaging combined with multi-photon microscopy or with the use of near infrared nanocrystals.

The large two-photon absorption cross section of QDs enables more efficient probing of thick specimens by multiphoton excitation microscopy [36••]. Using this technique, fluorescence signals could be detected hundreds of microns deep through the skin of live mice [20] and thick tissue specimens [37]. Stroh *et al*. [38] recently explored the use of QDs in anatomical imaging with multiphoton microscopy. Unlike traditional fluorescence labeled dextran vessel markers, the nanocrystals distinctly differentiate tumor vessels from perivascular cells and matrix. This group also assessed the ability of nanocrystals to monitor tumor and cell trafficking [38]. These findings show the potential uses of QDs in designing drug delivery particles and tumor pathophysiological studies.

Figure 3



QDs were used to study mixed cell interactions in a 3-D Matrigel culture system. **(a)** Human mammalian epithelial MCF 10A cells (labeled with green emitting silica coated QDs) form acini structures after growing in growth-factor reduced matrigel for 10 days. **(b)** After the acini were formed, human breast tumor MDA-MB-231 cells (labeled with red-emitting silica coated QDs) were added to the culture. After 14–16 h of incubation, the tumor cells had attached to the acini and the image was taken. **(c)** The contact was fatal to the tumor cells, which were found dead surrounding the MCF 10A acinus (see glossary). Most of the tumor cells had lysed, leaving transparent ghosts loosely attached to the acinus, but a few newly attached dead tumor cells still retained red-emitting QDs. **(d)** The MCF-10A acini and all invading tumor cells; it is a superimposition of all sections, displaying the sharp edge of each cell followed by a projection of color-coded depth information, so that red is the uninvolved lower portion of the MCF-10A acini and the tumor cells are shades of orange through blue. Bar = 10 μm. From manuscript in preparation with permission from CA Larabell.

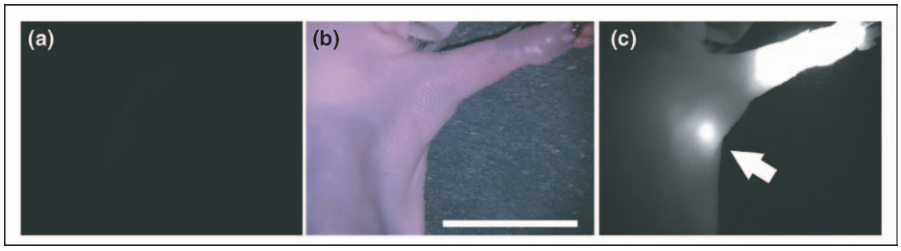
The tracking and imaging of nanocrystals in live animals has been achieved by Nie's group [34•]. QDs were conjugated to the antibody specific for the prostate cancer cell marker PSMA (prostate-specific membrane antigen). After injection into mice that had received a transplant of human prostate cancer cells, the quantum dot-tagged PSMA antibodies recognized and bound at the tumor site and were clearly imaged *in vivo*. Because of the large absorption coefficient and long lifetime, *in vivo* images of nanoparticles were much brighter and more sensitive than images with green fluorescent protein [34•].

One caveat to this approach in live animal imaging is the significant autofluorescence of the background. Several strategies can be applied to solve this problem. One approach is to use spectral imaging or emission scanning microscopy to separate the nanocrystal fluorescence signal from background noise [34•,37]. Because nanocrystals have narrow emission bands, this method also enables multicolor tracking of up to five different nanoparticles *in vivo* [37]. An even more effective solution is to move from visible light to near infrared (NIR), because most tissue chromophores absorb light weakly at such long wavelengths. Another advantage of NIR imaging is deeper penetration than that achieved with visible light. Kim and co-workers [11•] were the first to demonstrate the use of NIR QDs in their experiment to map sentinel lymph nodes (SLN) during surgical procedures (Figure 4). The nanoparticles, after intradermal injection into the animal, entered the lymphatic system and were followed using an intraoperative imaging system. The surgeon followed the flow of nanocrystals in real time with NIR image guidance, and quickly identified the position of the SLN in a precise and rapid surgical procedure. NIR nanocrystal imaging of blood vessels and the beating heart through 1–2 mm of skin and tissues have also been reported [39].

Semiconductor nanocrystals for neurobiology

One common approach to studying neurotransporters (see glossary) involves the use of radiolabeled substrates or antagonists that can be monitored with high sensitivity. However, the costs and complexity involved in using radiolabeled materials are high. Additionally, real time monitoring of the transporter activity is not possible. By contrast, a fluorescence-based approach enables the localization and direct monitoring of real-time activities.

Figure 4



Near infrared (NIR) QD sentinel lymph node mapping in the mouse. The mouse was injected intradermally with 10 pmol of NIR QDs in the left paw. **(a)** Pre-injection NIR autofluorescence image; **(b)** 5 min post-injection white light color video images; **(c)** 5 min post-injection NIR fluorescence image. An arrow indicates the putative axillary sentinel lymph node. Reproduced with permission from Kim *et al*. [11•].

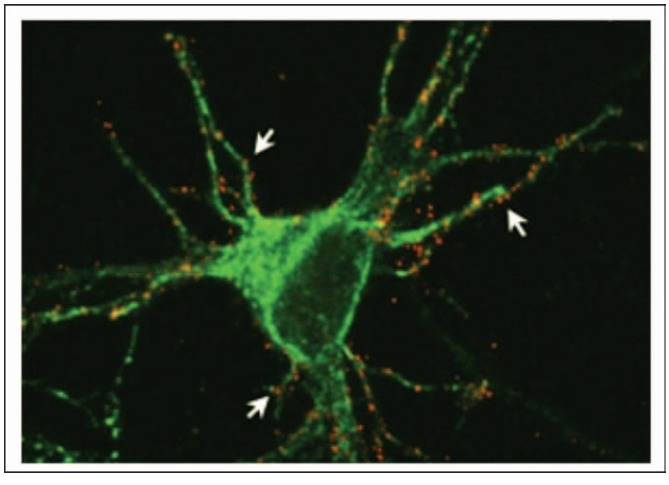
Owing to their high degree of photostability and brightness, QDs are more suitable probes than organic dyes for studies of neuronal protein or receptor dynamics over an extended period of time. QDs have been used to track individual glycine receptors, a major inhibitory neurotransmitter receptor, on the surface of cultured spinal neurons [10] (Figure 5). Compared with cyanine dye 3 (Cy3), fluorescent nanoparticles had a significantly higher signal-to-noise ratio and enabled tracking of single glycine receptors for at least 20 min, which is 200 times longer than that with Cy3 dye. Also, because of their small dimensions, nanoparticles are able to access dense synaptic regions and provide dynamic analysis that cannot be achieved with the use of 500 nm latex beads, one of the probes typically used for studying single molecule properties in live cells.

Besides single molecule studies of neurotransmitters, QDs have been used to study neurotransmitter localization and signal pathways. Nanocrystals conjugated with peptides, antibodies, or other small molecules recognize their target cell surface receptors [25,40–42]. In addition, nanocrystal probes, after binding to their targets, can modulate receptor functions by either inhibiting ligand transportation [41] or activating downstream signaling pathways [25,42].

Rosenthal *et al*. [41] used nanocrystals conjugated with the neurotransmitter serotonin to target serotonin transporters on transfected cells. Serotonin-labeled nanocrystals specifically interacted with the serotonin receptor, and inhibited the transportation of free serotonin in a manner similar to that of an antagonist.

Recently, Mason and co-workers [40] studied norepinephrine (NE) and dopamine (DA) transporter (NET and DAT) locations and activities with semiconductor nanocrystal linked antibodies and peptides. With streptavidin–biotin interaction, nanocrystals can specifically bind to NETs in transfected cells and surface protein

Figure 5



QDs as markers for glycine receptor (GlyR) localization in neurons. QD labeled GlyR (red) was detected over the somatodendritic compartment identified by microtubule-associated protein-2 (green). Arrows mark clusters of QD labeled GlyRs located on dendrites. Reproduced with permission from Dahan *et al*. [10].

limbic associated membrane protein (LAMP) in hippocampal cultures. Because the activity of the Ang II receptor is closely correlated with that of NET, the authors studied the localization of this receptor using nanocrystal–neuropeptide Ang II conjugates. In the future, this approach should enable study of Ang II receptor redistribution and dynamics in relation to NET activity in real-time.

To investigate whether QDs can function as fluorescent nano-devices to evoke specific cell physiological responses, Vu *et al.* [42] linked β the subunit of nerve growth factor (βNGF) to the nanocrystal surface and used this complex to target tyrosine kinase A (TrkA) receptors of PC12 cells. They reported that nanocrystal–βNGF activated TrkA receptor-initiated downstream signaling that resulted in conversion of PC12 cells to a neural phenotype.

These experiments show that QD–ligand conjugates are promising imaging probes for studying receptor-mediated activities and that they will have a wide range of applications in pharmaceutics and therapeutics.

Toxicity

Cadmium and selenium are known to be toxic [24]. Therefore, concerns have arisen about the toxicity and environmental impact of semiconductor nanocrystals. Most of the above cell and animal experiments showed that when correctly capped by both ZnS and hydrophilic shells, no obvious CdSe nanocrystal toxicity was observed under normal experimental conditions. Several groups have varied parameters such as synthesis, surface coating and incubation concentration to further investigate the potential toxicities of nanocrystals [24,43–45]. Cytotoxicity was observed when Cd^{2+} or Se^{2+} ions were released. This occurred when the nanoparticle surface coating was not stable, exposing the CdSe to oxidization by air or UV damage [24,45]. Surface molecules also have a role in QD cytotoxicity [44,45]. Although cells can tolerate PEG-silica coated QDs at concentrations up to 30 μM (QDs Cd surface atom concentrations), mercaptopropionic acid coated QDs have deleterious effect at ~6 μM [45].

Besides cytotoxicity, the degradation and metabolism of nanocrystals in the body remains to be investigated and there are reports that injected nanocrystals can accumulate in kidney, liver and spleen [34•,35]. Whether or not nanocrystals can ultimately be cleared from the body is not known. More research in this area must be completed before they can be used as probes for diagnostic applications.

Conclusions

In the past several years, there has been an increasing amount of interest in using QDs in an expanding variety of biological applications. Although they will not replace traditional fluorophores in biological imaging, QDs have been gradually accepted as better alternative probes with enhanced signal-to-noise ratios, extremely high stability, and improved specificity, factors that make them suitable for studying important biological problems. Owing to their relatively large size and heavy metal composition, QDs are potential candidates for high resolution imaging techniques, such as electron microscopy and x-ray microscopy. Doping QDs with other elements will also generate single probes for multi-scale imaging. With better control of QD synthesis, bioconjugation and toxicity promised by the future, it will hopefully be possible to generate QDs suitable for clinical diagnosis.

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- •• of outstanding interest

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